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# Fast and efficient measurement of the short term alcohol marker 5-hydroxytryptophol / 5-hydroxyindolic acid in urine with RP-HPLC and fluorescence detection

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**AIMS:** The increase of the ratio of 5-hydroxytryptophol / 5-hydroxyindolic acid (5-HTOL / 5-HIAA) in urine is a very specific and reliable marker for the detection of excessive drinking in the recent past, when blood or urine alcohol levels have already faded. Though this marker is known already for several years, its utility was limited by the complexity of its determination, which is most of the time done by HPLC with electrochemical detection. A new and much simpler, but still very specific and sensitive HPLC method has been developed using pre-column derivatisation of the 5-hydroxyindole (5-OH-Indole) groups and fluorescence detection.

**METHODS:** Glucuronides and Sulfates of 5-OH-indoles in urine samples are hydrolyzed by incubation with glucoronidase/sulfatase for 24 hours at 40°C. Their fluorescent derivatives are obtained by reaction with a basic 4-methylbenzylamine / $K_3[Fe(CN)_6]$ solution for 10 minutes. After neutralization with acetate buffer, the 20µl sample is directly applied onto the HPLC system.
For separation a 60 × 4mm column packed with Spherisorb ODS II 5µm was chosen with 15mM acetate buffer pH 3.5/acetonitrile (55:45) as mobile phase, giving a fast separation in less than 5 minutes. Because also other phenolic compounds out of the urine matrix may give (weak) derivatives which show up later in the chromatogram, the sample is loaded onto a short pre-column (4 × 4mm, RP8) which is exchanged for the injection loop instead of the injection valve. After injection the pre-column is switched into the eluent stream for only 30 sec and then washed under reverse flow. Fluorimetric detection at excitation: 340nm and emission: 450nm.

**RESULTS:** The derivatisation of the two 5-OH-indoles with benzylamine under oxidative conditions results in a fast formation of highly fluorescent derivatives which are stable for more than 24 hours. They have very good chromatographic properties and can be easily separated on a RP-column. Because of the high selectivity 5-HTOL and 5-HIAA can be directly determined in urine samples under these conditions down into sub-nanogram range without further sample extraction. Only 20µl of urine sample is necessary for analysis. Day-to-day and within-day precision is good in the dimension of 6-7% c.v. under routine running conditions.
Series of repeatedly collected urine samples from patients in alcohol withdrawal therapy were analyzed and showed a clear-cut increase of 5-HTOL / 5-HIAA ratios on those days, when a relapse had happened (>15 [pmoles/nmoles]).

**CONCLUSIONS:** The pre-column derivatisation of 5-HTOL and 5-HIAA with 4-methylbenzylamine allows the development of a very specific and sensitive determination of the two 5-OH-indoles in urine directly using reversed phase chromatography. To minimize analysis time, some late eluting peaks can be avoided with a simple column switching procedure. The method is very stable and is optimal for high throughput routine monitoring in patients to detect a recent alcohol misuse.

**KEYWORDS:** *Alcohol marker, 5-hydroxytryptophol, Pre-column derivatisation, HPLC, Column switching*

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